2010; Park *et al*, 2013). To our knowledge, the present study is the first to demonstrate the importance of NAADP as a Ca^{2+} signaling messenger in the differentiation of keratinocytes. The highly potent action of NAADP in keratinocyte differentiation suggests potential clinical applications for this Ca^{2+} signaling molecule.

All animal studies were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Identification of BRAF 3′UTR Isoforms in Melanoma

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TO THE EDITOR

BRAF protein kinase is a crucial player in melanoma, as it belongs to the highly oncogenic RAS/RAF/MEK/ERK signaling pathway (Matallanas *et al.*, 2011), is mutated in about 50% of melanoma cases (Cantwell-Dorris *et al.*, 2011), is causally linked to melanomagenesis (Dankort *et al.*, 2009) and has recently become a valuable therapeutic target against metastatic melanoma (Menzies and Long, 2014).

In the past years, the activity and regulation of BRAF protein have been studied extensively (Matallanas *et al.*, 2011). However, the regulation of BRAF expression, which in principle is equally important, has been largely neglected. Here, we show that *BRAF* mRNAs exist in at least two isoforms, which differ in the length and sequence of their 3′UTRs, and we discuss the implications that this discovery may have in terms of BRAF biology.

By performing 3′RACE in A375 melanoma cells, we obtained two most abundant PCR fragments, which have been cloned and sequenced (Figure 1a). The A fragment corresponds to the 121 nt long canonical (c) 3′UTR reported for human *BRAF* in the most common databases (Ensembl, ENST00000288602; NCBI, NM_004333.4, polyA site at nt. 2442-47; Figure 1b, upper, Supplementary Figure S1a and Supplementary Note S1 online). Conversely, the 1352 nt long fragment B corresponds to a *BRAF* tv that so far has been only predicted (XM_005250045.1 (*BRAF* tv X1), XM_005250046.1 (*BRAF* tv X2), ENST00000496384). Interestingly, this *BRAF* tv, which from now on we call X1, is transcribed from an additional 19th exon located ~8 kbp downstream of the last canonical exon (the 18th) and is highly conserved between humans, mouse, and rat (Figure 1b, lower, Supplementary Figure S1b and Supplementary Note S2 online).

In order to confirm the results obtained by 3′RACE, we used real-

Abbreviations: ceRNA, competitive endogenous RNA; ORF, open reading frame; MRE, microRNA recognition element; RNA-seq, RNA-sequencing; RBP, RNA binding protein; tv, transcript variant

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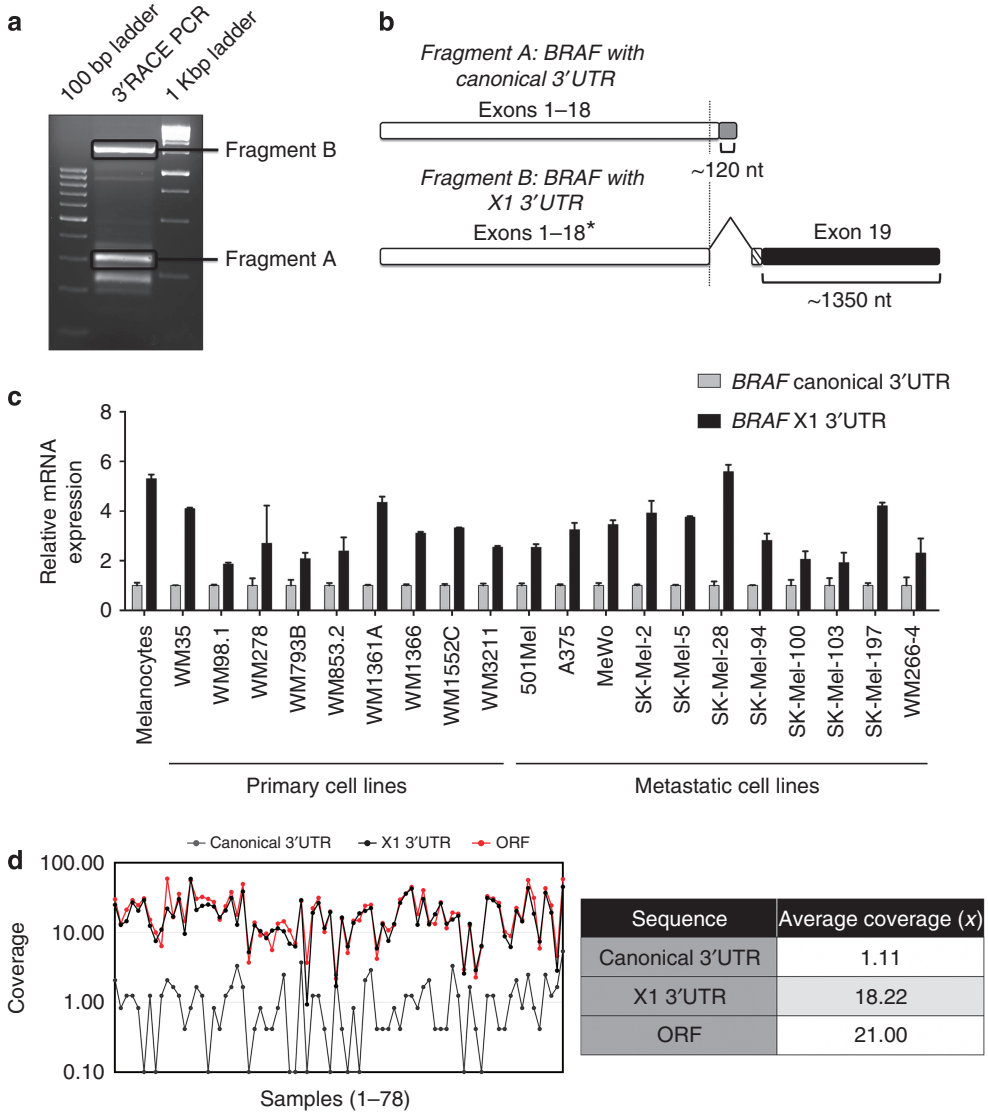
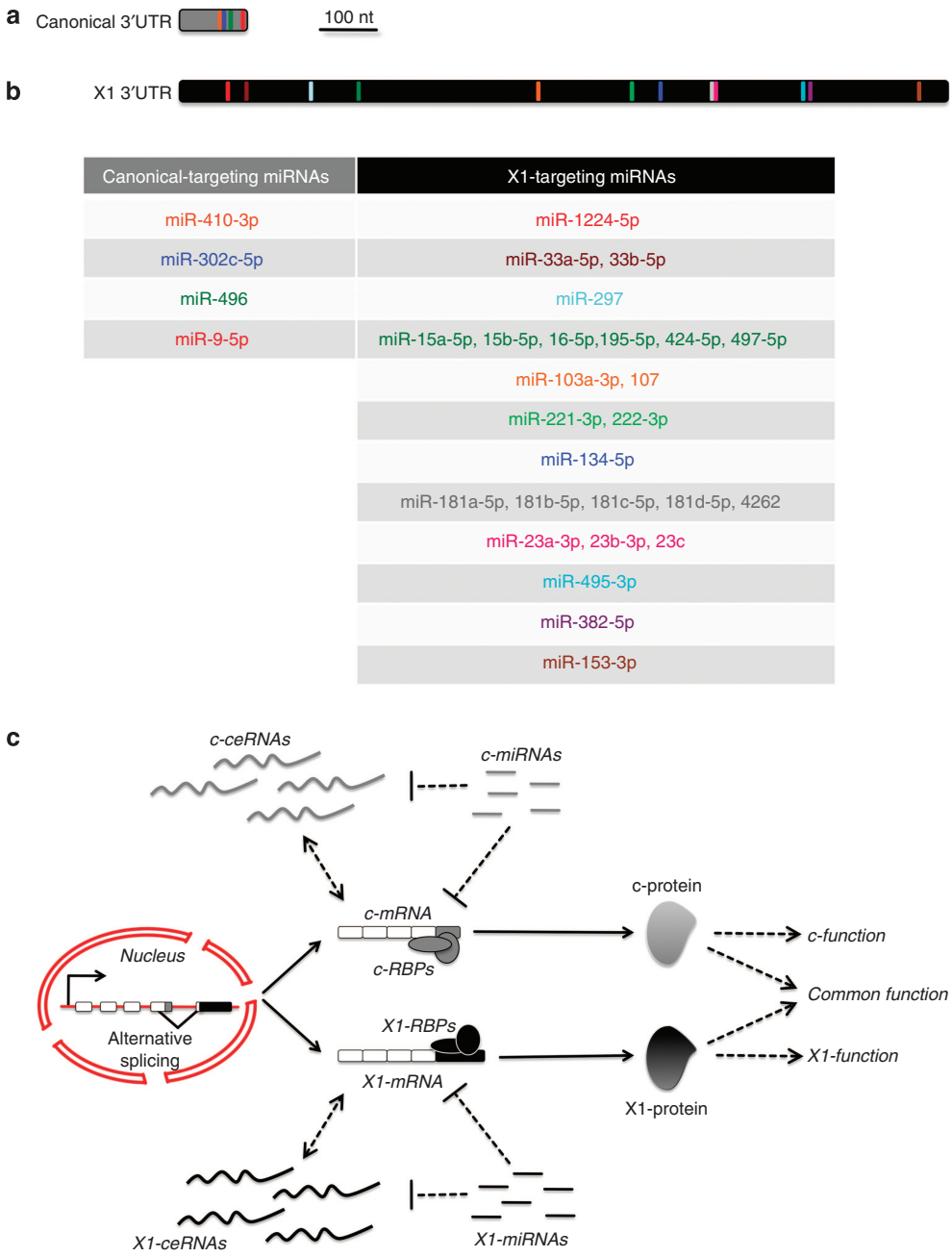


Sequence	Average coverage (*x*)
Canonical 3′UTR	1.11
X1 3′UTR	18.22
ORF	21.00

Figure 1. Identification of *BRAF* 3′UTR isoforms. (**a**) 3′RACE performed on the cDNA derived from A375 melanoma cells produced two most abundant PCR fragments (A and B). (**b**) Schematic representation of 3′RACE results. Fragment A corresponds to the canonical *BRAF* 3′UTR, which is 121nt long and is transcribed from exon 18. Fragment B corresponds to the predicted X1 3′UTR, which is 1352 nt long and is transcribed from an additional exon 19. The sequences of the two 3′UTRs are completely unrelated. The very last parts of the ORFs differ as well. White rectangles: coding sequences. Gray and black rectangles: canonical 3′UTR and X1 3′UTR, respectively. Striped rectangle: X1-specific coding sequence. (**c**) Quantification of the canonical and the X1 3′UTR by real-time PCR. The bars represent the mean ± s.e.m. of two independent experiments. (**d**) Coverage of the canonical 3′UTR, the X1 3′UTR, and the ORF of *BRAF* in 78 melanoma samples (SKCM data set, The Cancer Genome Atlas).

time PCR and measured the expression levels of the c and the X1 isoforms in melanocytes and in a wide panel of melanoma cell lines (Figure 1c). Because of the high similarity between *BRAF* and its expressed pseudogene *BRAFP1* (Zou *et al.*, 2009; Kalyana-Sundaram *et al.*, 2012), which spans the entire coding sequence and extends to the canonical 3′UTR, it was not possible to design real-time primers that are c specific (that is, not cross amplifying the X1 isoform) and at the same time *BRAF* specific (that is, not cross amplifying *BRAFP1*, see Supplementary Figure S2a,b online). Therefore, we cannot exclude the fact that the PCR products obtained using the c-specific primers are derived from the amplification of both the canonical parental *BRAF* and *BRAFP1* (see Supplementary Note S3 online for further details). However, even taking this technical limitation into account, we still found that the X1 isoform is consistently more expressed compared with the c isoform. This trend is shared between normal melanocytes and melanoma lines with different BRAF and NRAS mutational status. Furthermore, a similar trend is observed in other lineages (breast, cervix, colon, lung, and prostate, Supplementary Figure S3 online).



Canonical-targeting miRNAs	X1-targeting miRNAs
miR-410-3p	miR-1224-5p
miR-302c-5p	miR-33a-5p, 33b-5p
miR-496	miR-297
miR-9-5p	miR-15a-5p, 15b-5p, 16-5p,195-5p, 424-5p, 497-5p
	miR-103a-3p, 107
	miR-221-3p, 222-3p
	miR-134-5p
	miR-181a-5p, 181b-5p, 181c-5p, 181d-5p, 4262
	miR-23a-3p, 23b-3p, 23c
	miR-495-3p
	miR-382-5p
	miR-153-3p

Figure 2. 3′UTR-specific *BRAF*-targeting microRNAs. (**a**) Position of predicted microRNA recognition elements on the canonical (gray) and X1 (black) 3′UTRs. (**b**) List of canonical and X1-predicted microRNAs. (**c**) Schematic representation of *BRAF* isoforms and of their implications for BRAF expression and function. Alternative splicing produces two *BRAF* transcripts (canonical and X1) that vary in the very last part of the ORF and in the 3′UTR. The c- and X1-*BRAF* mRNAs are expected to be implicated in distinct regulatory circuits at the post-transcriptional level. Analogously, the c- and X1-BRAF proteins are expected to have both common and isoform-specific functions. The color gray refers to the canonical 3′UTR, whereas the black color refers to the X1 3′UTR. Rectangles: *BRAF* coding sequence and 3′UTR; curved lines: *BRAF*-related ceRNAs; straight lines: *BRAF*-regulating microRNAs; solid figures: proteins; double red lines: nuclear membrane.

In order to unambiguously detect both the c and the X1 isoforms, we analyzed the RNA-sequencing data of 78 melanoma samples that are available at The Cancer Genome Atlas (SKCM data set). As reported in Figure 1d and Supplementary Figure S4 online, we confirmed that the X1 isoform (black) is more expressed compared with the c isoform (gray). Furthermore, the comparison of the expression level of *BRAF* ORF (red) with that of the X1 3′UTR (black) allowed us to infer that the X1 isoform accounts indeed for the vast majority of the *BRAF* molecules.

The finding that *BRAF* 3′UTR is not just one but rather a mix of multiple transcripts of different length and sequence

opens entirely new fields of investigation, as it calls for a deeper analysis of the regulation of BRAF expression and function at multiple levels.

First and foremost, as c and *X1 BRAF* have completely unrelated 3′UTR sequences, they are expected to be regulated post-transcriptionally by different sets of RNA binding proteins and microRNAs. In Figures 2a and b we indeed report that the four microRNAs predicted as c 3′UTR-targeting and the 12 microRNA families predicted as X1 3′UTR-targeting do not show any overlap.

Along this line, it should also be considered that different 3′UTRs, hence different sets of bound microRNAs, might imply the involvement of the two *BRAF* isoforms in different competitive endogenous RNA networks (Tay *et al.*, 2014). As mentioned above, the 3′UTR of *BRAFP1* pseudogene is highly similar to the canonical 3′UTR (Supplementary Figure S2b online). Therefore, as far as the 3′UTR is concerned, *BRAFP1* is supposed to function as a competitive endogenous RNA only for c *BRAF* and not for *X1 BRAF* (Poliseno *et al.*, 2010; Supplementary Figure S2c online).

Aside from the 3′UTR sequence, also the very last part of *BRAF* ORF is different between the c and the X1 isoform (Supplementary Figure S5 online, AFPVH vs EFAAFK). Therefore, those cells that express *BRAF* transcripts that differ in their 3′UTRs, also express BRAF proteins that differ in their C-term domains, with all the implications that this might have in terms of the kinase-dependent and -independent activities of BRAF, as well as of its post-translational regulation (Lee *et al.*, 2011; Matallanas *et al.*, 2011; Chen *et al.*, 2012).

The alternative splicing of *BRAF* exons is quite common. Depending on the exons that are skipped, a kinase-dead protein (Hirschi and Kolligs, 2013), a constitutively active protein (Baitei *et al.*, 2009), or a protein insensitive to BRAF inhibitors (Poulikakos *et al.*, 2011) is translated. Our discovery of a 19th exon enhances the relevance of alternative splicing, as it implies that it might be used to regulate not only the activity of BRAF protein, but also the level of *BRAF* mRNA isoforms (Figure 2c).

As a final remark, we would like to emphasize that an in-depth study of the regulation of *BRAF* expression, besides being relevant *per se*, could also have important translational implications, as it could help improve the current therapeutic approaches against metastatic melanoma (Kwong and Davies, 2014; Menzies and Long, 2014).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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